PRIMER FOR DETECTION OF HUMAN PAPILLOMAVIRUS

Technical Field

The present invention relates to primers specific to the genome of human papillomavirus (hereinafter, referred to as "HPV"), a kit for detecting the HPV genome comprising the primers, and a method of detecting the HPV genome using the primers.

Background Art

HPV is a double-stranded DNA virus whose circular genome is approximately 8 kb long. HPV inhabits the vagina, and the infection thereof is hard to treat and is not easily made to disappear. HPV infects the epithelial cells of other mammals as well as humans, and generally induces warts, and sometimes malignant tumors, at the site of infection. HPV is detected in over 90% of condyloma accuminata cases (enlarged warts having a cauliflower-like appearance around the genitals or the anus) and almost 100% of cervical cancer cases. In particular, cervical cancer accounts for 22.1% of all cancers found in women in Korea, and is the second leading cause of cancer death among women.

Thus, establishing a method of effectively detecting HPV, which causes cervical cancer, is important for the

diagnosis, prophylaxis and therapy of the disease. Also, HPV needs to be effectively detected to evaluate the efficacy and toxicity of a vaccine against HPV after vaccination.

A nucleic acid-based test for diagnosing an infectious disease employs a standard method of isolating nucleic acids from individuals and clinical materials. Since target DNA or RNA is present in clinical specimens in small amounts, several major techniques used in diagnostic laboratories are based on signal amplification and target amplification. These methods aid detection, are useful in the identification of individuals without culture, and contribute to the treatment as well as diagnosis of infectious diseases. PCR, which is a nucleic acid amplification technique (NAT), is widely used because it enables the selective amplification of specific targets, present in low concentrations, to detectable levels. In addition to the qualitative detection of viruses, quantitative determination of viral load in clinical specimens is now realized to be of great importance with respect to the diagnosis, prognosis, and therapeutic monitoring of HPV infection (Pfaller M.A, *Emer. Infect. Dis.* 7, 2, 2001).

The genome of all types of HPV is divided into two major regions: early and late regions. The early region of about 4.5 kb codes for genes which are associated with

functions including viral DNA replication (E1), induction or suppression of the action of DNA encoding a protein inducing malignant transformation of host cells (E2), synthesis of proteins responsible for the growth of host cells and viruses (E4), stimulation of the activity of epidermal growth factor (EGF) and colony stimulator factor (CSF) receptors (E5), and malignant transformation through permanent survival of cells, activation of oncogenes and inactivation of tumor suppressor genes (E7). In particular, the oncogenic E6 and E7 proteins, which are expressed after HPV infects the epithelial cells of a host, bind to tumor suppressor proteins of host cells, p53 and pRB, respectively, thereby inhibiting the function of the tumor suppressor proteins, leading to the transformation of infected cells, resulting in the development of tumors. The late region of 2.5 kb comprises genes coding for viral major (L1) and minor (L2) capsid proteins and a non-coding region of 1 kb, which is called the long control region (LCR) that regulates the transcription and translation of the two late genes.

With recent rapid advances in molecular biological techniques, the genetic structure of HPV has been identified, revealing genomic sequences of many genotypes of HPV. HPV is classified according to the difference in DNA sequences of E6, E7 and L1 open reading frames (ORFs). When the nucleotide sequences of the ORFs differ by more

than 10%, an HPV is assigned a new genotype. HPV subtypes differ by 2% to 10%, and HPV variants differ by less than 2%.

In order to specifically detect high risk HPV types 16, 18 and 31 and a low risk HPV type 11 among a large number of HPV genotypes, which are detected in tissues of cervical cancer and carcinoma in situ, respectively, the present inventors intended to detect a gene specific to each genotype of these viruses, and selected the L1 gene as such a gene.

In order to specifically detect the HPV L1 gene, the present inventors determined the sequences of L1 genes of the HPV types 11, 16, 18 and 31, which are specifically found in Koreans, and constructed primers capable of specifically binding to the L1 gene of each HPV type. The present inventors found that when PCR was performed with the primers, each HPV genotype can be specifically detected and can be precisely quantified down to very low amounts, thereby leading to the present invention.

Disclosure of the Invention

It is therefore an object of the present invention to provide a primer pair selected from among pairs of primers capable of complementarily binding to the genome of human papillomavirus (HPV) and having nucleotide sequences

represented by SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8.

It is another object of the present invention to provide a method of detecting the HPV genome, which is based on performing a polymerase chain reaction (PCR) for DNA contained in a biological sample using one or more primer pairs selected from among pairs of primers capable of complementarily binding to the HPV genome and having nucleotide sequences represented by SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8.

It is a further object of the present invention to provide a kit for detecting the HPV genome, comprising one or more primer pairs selected from among pairs of primers capable of complementarily binding to the HPV genome and having nucleotide sequences represented by SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8.

Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 schematically represents the construction of

recombinant plasmids constructed with amplified L1 genes of HPV genotypes 11, 16, 18 and 31 (A), and also shows the results of restriction enzyme mapping of the recombinant plasmids (B);

Fig. 2 is an alignment of HPV 16 L1 sequences;

Fig. 3 is an alignment of HPV 31 L1 sequences;

Fig. 4 is an alignment of HPV 11 L1 sequences;

Fig. 5 is an alignment of HPV 18 L1 sequences;

Fig. 6 is a multiple alignment of nucleotide sequences of L1 genes of HPV genotypes 11, 16, 18 and 31 and the consensus L1 sequence;

Fig. 7 shows the results of a sensitivity test using plasmid DNA templates, each of which carry the HPV 11, 16, 18 or 31 L1 gene;

Fig. 8 shows the results of a differentiality test using plasmid DNA templates, each of which carries the HPV 11, 16, 18 or 31 L1 gene;

Fig. 9 shows the results of heat stability and long-term preservation tests for HPV L1 plasmids after storage for 3 weeks;

Figs. 10 and 11 show the results of heat stability and long-term preservation tests for HPV L1 plasmids after storage for 15 weeks; and

Fig. 12 shows the results of an applicability test using predetermined amounts of HPV L1 plasmids supplemented with various DNA backgrounds.

Best Mode for Carrying Out the Invention

In one aspect, the present invention relates to primers capable of complementarily binding to the HPV genome.

In a detailed aspect, the present invention relates to a primer pair selected from among pairs of primers capable of complementarily binding to the HPV genome and having the nucleotide sequences represented by SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8.

The term "primer", as used herein, refers to a short nucleic acid sequence having a free 3' hydroxyl group, which is able to undergo base-pairing interaction with a complementary template and serves as a starting point for replicating the template strand. A primer is able to initiate DNA synthesis in the presence of a reagent for polymerization and four different nucleoside triphosphates in suitable buffers and at a suitable temperature. With respect to the objects of the present invention, primers specifically amplify a specific region of the L1 gene of HPV 16, described in Fig. 2, HPV 31, described in Fig. 3, HPV 11, described in Fig. 4, and HPV 18, described in Fig. 5. Thus, the primers of the present invention consist of a pair of sense and antisense primers having a sequence of 7 to 50 nucleotides, and more preferably 10 to 30

nucleotides, the sequence capable of complementarily binding to the aforementioned HPV L1 gene. In detail, a specific region of the HPV L1 gene may be specifically amplified with a pair of primers having the nucleotide sequences of SEQ ID Nos. 1 and 2 for HPV 11, a pair of primers having the nucleotide sequences of SEQ ID Nos. 3 and 4 for HPV 16, a pair of primers having the nucleotide sequences of SEQ ID Nos. 5 and 6 for HPV 18, and a pair of primers having the nucleotide sequences of SEQ ID Nos. 7 and 8 for HPV 31.

The primers of the present invention may be chemically synthesized using a phosphoramidite solid support method or other widely known methods. These nucleic acid sequences may also be modified using any means known in the art. Non-limiting examples of such modifications include methylation, capsulation, replacement of one or more native nucleotides with analogues thereof, and inter-nucleotide modifications, for example, modifications to uncharged conjugates (e.g., methyl phosphonate, phosphotriester, phosphoroamidate, carbamate, etc.) or charged conjugates (e.g., phosphorothioate, phosphorodithioate, etc.). Nucleic acids may contain one or more additionally covalent-bonded residues, which are exemplified by proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalating agents (e.g., acridine, psoralene, etc.),

chelating agents (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylating agents. The nucleic acid sequences of the present invention may also be altered using a label capable of directly or indirectly supplying a detectable signal. Examples of such a label include radioisotopes, fluorescent molecules, and biotin.

When PCR was performed with the primers provided in the present invention, which have the nucleotide sequences of SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8, the primers were found to be able to specifically detect each of the four different HPV genotypes and to be sensitive enough to amplify as few as 62.5 copies of a plasmid.

Thus, the present primers may be useful in the detection of HPV infections, the identification of infective HPV genotypes, the epidemiological evaluation of HPV, the effectiveness and toxicity of developed HPV vaccines, and the like.

In another aspect, the present invention provides a method of detecting the HPV genome, which is based on performing a polymerase chain reaction (PCR) for DNA contained in a biological sample using one or more primer pairs selected from among pairs of primers capable of complementarily binding to the HPV genome and having the nucleotide sequences represented by SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7

and 8.

The term "biological sample", as used herein, includes, but is not limited to, samples, such as tissues, cells, whole blood, sera, plasma, saliva, sputa, cerebrospinal fluid, urine, or the like, of individuals infected with HPV or suspected of being infected with HPV, or individuals vaccinated with a HPV vaccine.

A method for identifying the presence and genotype of HPV is particularly not limited as long as it employs the aforementioned primers. Examples of such methods include direct identification of HPV DNA using a primer of a specific strand as a probe, Southern blotting, dot blotting, and filter in situ hybridization (FISH). Alternative methods include a method based on amplifying HPV DNA using a pair of primers, genotype-specific polymerase chain reaction (PCR), and general-primer PCR. PCR is most preferred.

The term "polymerase chain reaction (PCR)", as used herein, is a representative nucleic acid amplification technique (NAT), which enzymatically amplifies a specific DNA region of interest in vitro. The PCR method, which was developed in 1985 by Mullis et al., can amplify any segment of a DNA molecule if its boundary sequences are known. PCR basically consists of three major steps: denaturation, annealing and extension. A specific DNA sequence is amplified while these three steps are repeated. In the

first step (denaturation) of PCR, a double-stranded template DNA is denatured into two single strands. In the second step (annealing), primers anneal with the two kinds of single-stranded DNA, in which a sequence desired to be amplified is interposed between the primer binding regions. In the third step (extension), a heat-resistant DNA polymerase extends the primers and synthesizes the complementary strand of the target sequence. This cycle is repeated 25 to 30 times.

Primers are the most important factor determining the reliability of PCR results. Some primer sequences can give rise to non-specific amplification, leading to false results. In this regard, the present invention provides reliable primer pairs. The performance of PCR with the primer pairs of the present invention enables accurate detection of HPV genotypes and sensitive quantitative analysis of very small amounts. Also, when PCR is carried out with the primer pairs of the present invention, consistent results are obtained upon repeated PCR performance. That is, since the primer pairs of the present invention are highly valid and reliable, the results obtained with the present primer pairs are highly reliable.

In a preferred aspect, the present invention provides a method of detecting the HPV 11 L1 gene using a pair of primers having the nucleotide sequences of SEQ ID Nos. 1 and 2.

In another preferred aspect, the present invention provides a method of detecting the HPV 16 L1 gene using a pair of primers having the nucleotide sequences of SEQ ID Nos. 3 and 4.

In a further preferred aspect, the present invention provides a method of detecting the HPV 18 L1 gene using a pair of primers having the nucleotide sequences of SEQ ID Nos. 5 and 6.

In yet another preferred aspect, the present invention provides a method of detecting the HPV 31 L1 gene using a pair of primers having the nucleotide sequences of SEQ ID Nos. 7 and 8.

A PCR for amplifying an HPV gene, in detail the L1 gene, using the primers of the present invention may be carried out through an ordinary PCR method. Also, conditions including time, temperature and cycle number, under which denaturation, annealing and extension reactions are allowed to occur, may vary. In the present invention, PCR conditions included 35 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 1 min or 1 min 30 sec.

In a further aspect, the present invention provides a kit for detecting the HPV genome, comprising one or more primer pairs selected from among pairs of primers capable of complementarily binding to the HPV genome and having the nucleotide sequences represented by SEQ ID Nos. 1 and 2,

SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8.

In addition to the primer pairs, the detection kit of the present invention is composed of one or more different compositions, solutions or instruments, which are suitable for analysis methods. Preferably, the kit of the present invention includes the following constituents: a container containing detection primers; amplification reaction tubes or other suitable containers; reaction buffer (pH and magnesium concentration of which may vary); dNTPs; an enzyme such as Taq-polymerase; RNase; and sterile water. More preferably, the kit may further include a plasmid carrying an HPV gene as a positive control in order to realize quantitative analysis. Such a plasmid may be one or more selected from among pGEM-HPV11 L1, pGEM-HPV16 L1, pGEM-HPV18 L1, and pGEM-HPV31 L1, which will be described in the following examples.

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: Construction of recombinant HPV L1 plasmids (standard DNA)

PCR primers specific to low risk HPV 11 and high risk

HPV 16, 18 and 31 were designed based on major protein (HPV L1) sequences of the different genotypes of HPV, which are deposited in GenBank. In order to obtain HPV genotypes commonly found in Korean, tissues of Korean cervical cancer patients were obtained from clinical hospitals, and genomic DNA as an HPV genome source was extracted from the tissues. Biological tissue samples were paraffin sections or biopsy samples prepared for pathological examination. PCR was carried out using the extracted genomic DNA with primers having the nucleotide sequences of SEQ ID Nos. 9 to 16, which are listed in Table 1, below. As a result, PCR products of about 1.6 kb were obtained.

TABLE 1

Primer sequences against the HPV L1 gene for the preparation of national (Korean) standard DNA

HPV genotype	PCR fragment length	PCR primer sequence	
HPV 16	1596 bp	Sense	5'-GCCCCCAAGCTTGCCGCCACCATGCAGGTGACTTTTATTTACATCC-3' (SEQ ID No. 9)
		Anti-sense	5'-ATCGGGCTCGAGCAGCTTACGTTTTTTGCGTTTAGC-3' (SEQ ID No. 10)
HPV 18	1707 bp	Sense	5'-GCCCCCAAGCTTGCCGCCACCATGTGCCTGTATACACGG-3' (SEQ ID No. 11)
		Anti-sense	5'-ATCGGGGAATTCCTTCCTGGCACGTACACGCACACG-3' (SEQ ID No. 12)
HPV 31	1515 bp	Sense	5'-GCCCCCAAGCTTGCCGCCACCATGTCTCTGTGGCGGCCTAGC-3' (SEQ ID No. 13)
		Anti-sense	5'-ATCGGGGAATTCCTTTTTAGTTTTTTTACGTTTTGCTGGTGTAGTGG-3' (SEQ ID No. 14)
HPV 11	1506 bp	Sense	5'-GCCCCCAAGCTTGCCGCCACCATGTGGCGGCCTAGCGACAGC-3' (SEQ ID No. 15)
		Anti-sense	5'-ATCGGGGAATTCCTTTTTGGTTTTGGTACGTTTTCGTTTGGG-3' (SEQ ID No. 16)

PCR was carried out under the following conditions. PCR was carried out using the DNA samples extracted from tissues from patients as templates with 2.5 mM dNTP, reaction buffer, primer pairs (20 pmol) listed in Table 1, and SuperTaq Plus. According to the optimal annealing temperature of primers, a cycle of denaturation at 94°C for 1 min, annealing at Ta for 1 min, and extension at 72°C for 1 min 30 sec was repeated thirty five times, followed by final extension at 72°C for 10 min. Each PCR product was cloned into pGEM-T-Easy vector (Promega, USA) and transformed into *E. coli* DH5α. Plasmid DNA was then isolated and digested with EcoRI to determine whether the PCR product was successfully inserted (Fig. 1).

The nucleotide sequences of the PCR products were determined and compared with previously known nucleotide sequences coding for the L1 protein of HPV genotypes. HPV 16 was compared with AF402678, HPV 31 with J04353, HPV 11 with NC_001525, and HPV 18 with NC_001357. As a result, the nucleotide sequences coding for the L1 protein of the four HPV genotypes, which were identified according to the procedure described above, were found to be highly similar to the conventionally known nucleotide sequences coding for L1 proteins of the HPV genotypes.

EXAMPLE 2: Large preparation and quantification of the recombinant HPV L1 plasmids

E. coli cells transformed with the recombinant HPV L1 plasmids prepared in Example 1 were inoculated in 10 ml of LB medium supplemented with ampicillin, and grown in a shaking incubator at 37℃ overnight. Plasmid DNA was then isolated using an alkaline lysis method, and precisely quantified using a spectrophotometer. The plasmid copy number was calculated according to Equation 1, below.

[Equation 1]

Copy number of 1 kb fragment = (1000 bp × 660 g/mole) / (6.023 × 10^{23} molecules) = 1 × 10^{-18} g (1 fg)

Equation 1 means the following. One copy of a 1 kb (1000 bp) plasmid weighs 1 × 10^{-18} g (1 fg), and one gram of 1 kb plasmid DNA contains 10^{18} copies of the plasmid.

Using Equation 1, the number of copies of each of different HPV genotype plasmids was calculated, and a plasmid solution having 2000 copies was serially diluted two-fold, thereby yielding 10-㎕ solutions containing 2000, 1000, 500, 250, 125 and 62.5 copies of the plasmids.

EXAMPLE 3: Evaluation of the sensitivity of specific primers using the recombinant HPV L1 plasmids

Using the 10 ㎕ plasmid solutions containing 2000,

1000, 500, 250, 125 and 62.5 copies, prepared in Example 2, PCR was carried out. As a result, PCR primers displayed sensitivity in a manner dependent on the number of plasmid copies (indicating that the 10 μl DNA solutions respectively contained 2000, 1000, 500, 250, 125 and 62.5 copies of the plasmids). Referring to Fig. 6, PCR primers represented by SEQ ID Nos. 1 to 8 (Table 2) were determined.

TABLE 2

Primers for detecting the HPV L1 gene

Genotype	Sense primer	Anti-sense primer
HPV 11	TTAGGCGTTGGTGTTAGTGG (SEQ ID No. 1)	AAAATTCATAGCACCAAAGC (SEQ ID No. 2)
HPV 16	TTAGGTGTGGGCATTAGTGG (SEQ ID No. 3)	AAAGTCCATAGCACCAAAGC (SEQ ID No. 4)
HPV 18	TTAGGTGTTGGCCTTAGTGG (SEQ ID No. 5)	AAAGTCCATGGCACCATATC (SEQ ID No. 6)
HPV 31	TTAGGTGTAGGTATTAGTGG (SEQ ID No. 7)	AAAATCCATAGCTCCAAAGC (SEQ ID No. 8)

PCR was carried out as follows. 5 μl of 2.5 mM dNTP was mixed with 5 μl of 10 × buffer, primers (20 pmol) of SEQ ID Nos. 1 to 8, 0.5 μl of Taq polymerase, and distilled water to give a final volume of 40 μl. The mixture was supplemented with 10 μl of each template, thereby yielding a PCR mixture. PCR conditions included 35 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. PCR products were then separated on a 1.5% agarose gel for 40 min, and stained with ethidium bromide (EtBr). Band intensity was measured

using the software, Quantity One (Bio-Rad). Then, a regression function was derived in order to determine the relationship between band intensity and the number of plasmid copies, and a relative coefficient R was calculated to determine whether it was greater than 0.9. As a result of the sensitivity test for the PCR amplification method, the band intensity, as shown in Fig. 7, decreased in all of the four HPV genotypes in a manner dependent on the number of plasmid copies, and this PCR method was found to have a sensitivity detecting as few as 62.5 copies of the HPV L1 plasmids. When DNA was run on an agarose gel to determine the relationship between band intensity and the number of plasmid copies, the relative coefficient, as shown in Fig. 7, was greater than 0.9. These results indicate that a plasmid copy number test using the method of the present invention provides reliable results.

EXAMPLE 4: Evaluation of the specificity of primers using the recombinant HPV L1 plasmids

In order to determine whether the primers used in Example 3 specifically amplify each HPV genotype, the primer sets to the four different HPV genotypes were evaluated for whether they differentially amplify L1 templates of different HPV genotypes. PCR was carried out under the same conditions as in Example 3 except that the

templates were used in a concentration of 1000 copies. PCR was performed with each primer set using each of the four different HPV genotypes as a template. As a result of the PCR with each primer set using 1000 copies of each HPV genotype as a template, all primer sets to HPV 11, 16, 18 and 31 were found to specifically amplify only their corresponding templates (Fig. 8). The results, specifically that the primers of the present invention precisely detect only their corresponding HPV genotypes under optimized PCR conditions, indicate that the different primer sets enable HPV genotyping of clinical samples and differential detection of high risk HPV types 11, 16 and 18, infections of which are most likely to progress to cervical cancer. Thus, the present primers may become a very useful means of clinical diagnosis.

EXAMPLE 5: Evaluation of the heat stability and long-term preservation of the recombinant HPV L1 plasmids

To evaluate the heat stability and long-term preservation of primers, first, 30 $\mu\ell$ of 1000 copies of each HPV L1 plasmid was aliquotted into fifteen DNase/RNase-free vials. The containers were stored at 4℃, 22℃ and 37℃ for a heat stability test, and at -80℃ for long-term storage. Every three weeks, one vial at each storage temperature was subjected to a sensitivity test, which was carried out

according to the same procedure as in Example 3. The PCR with plasmids stored at 22℃ and 37℃ for three weeks showed negative results, indicating that standard DNA has a very low stability when stored at 22℃ and 37℃ (Fig. 9). In contrast, standard DNA stored at 4℃ and -80℃ for 15 weeks still provided highly sensitive PCR results, indicating that the standard DNA of the present invention is stable when stored at -80℃ for a long period of time (Figs. 10 and 11).

EXAMPLE 6: Evaluation of the applicability of primers using the recombinant HPV L1 plasmids

The procedure with the HPV L1 templates and primers provided in the present invention was evaluated to determine whether it could detect the HPV genome in human clinical samples, as follows. Genomic DNA was extracted from human rhabdomyosarcoma (RD), HeLa and SLK cells using a genomic DNA extraction kit (Qiagen), and the concentration thereof was determined using a spectrophotometer. Each DNA solution was diluted to concentrations of 10 ng and 100 ng. 100 ㎕ of each dilution was aliquotted and stored at -20℃. Then, PCR was carried out with 100 ng and 1 ㎍ of genomic DNA background using the same templates as in the sensitivity test of Example 3. PCR products were analyzed according to the same method used

for the sensitivity test. In the PCR with the genomic DNA background, the HPV templates, as shown in Fig. 12, could be detected even with as few as 62.5 copies, as in the absence of human genomic DNA. The PCR with genomic DNA background from HeLa cells, which contain the HPV 18 genome, exhibited positive results in all lanes.

Industrial Applicability

As described hereinbefore, the primer pairs specific to the L1 gene of HPV 11, 16, 18 and 31 according to the present invention may be useful in the detection of HPV infections, the identification of infected HPV genotypes, evaluation of the effectiveness and toxicity of developed HPV vaccines, and the like.

Claims

1. A primer pair selected from among pairs of primers capable of complementarily binding to human papillomavirus (HPV) genome and having nucleotide sequences represented by SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8.

2. A kit for detecting human papillomavirus (HPV) genome, comprising one or more primer pairs selected from among pairs of primers capable of complementarily binding to the HPV genome and having nucleotide sequences represented by SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8.

3. A method of detecting human papillomavirus (HPV) genome, comprising performing a polymerase chain reaction for genomic DNA contained in a biological sample using one or more primer pairs selected from among pairs of primers capable of complementarily binding to the HPV genome and having nucleotide sequences represented by SEQ ID Nos. 1 and 2, SEQ ID Nos. 3 and 4, SEQ ID Nos. 5 and 6, and SEQ ID Nos. 7 and 8.

4. The method as set forth in claim 3, wherein an HPV 11 L1 gene is detected using the primer pair having the

nucleotide sequences represented by SEQ ID Nos. 1 and 2.

5. The method as set forth in claim 3, wherein an HPV 16 L1 gene is detected using the primer pair having the nucleotide sequences represented by SEQ ID Nos. 3 and 4.

6. The method as set forth in claim 3, wherein an HPV 18 L1 gene is detected using the primer pair having the nucleotide sequences represented by SEQ ID Nos. 5 and 6.

7. The method as set forth in claim 3, wherein an HPV 31 L1 gene is detected using the primer pair having the nucleotide sequences represented by SEQ ID Nos. 7 and 8.

8. The kit for detecting the HPV genome as set forth in claim 2, further comprising one or more plasmids selected from among pGEM-HPV11 L1, pGEM-HPV16 L1, pGEM-HPV18 L1 and pGEM-HPV31 L1 as a positive control in order to realize quantitative analysis.

Fig. 1

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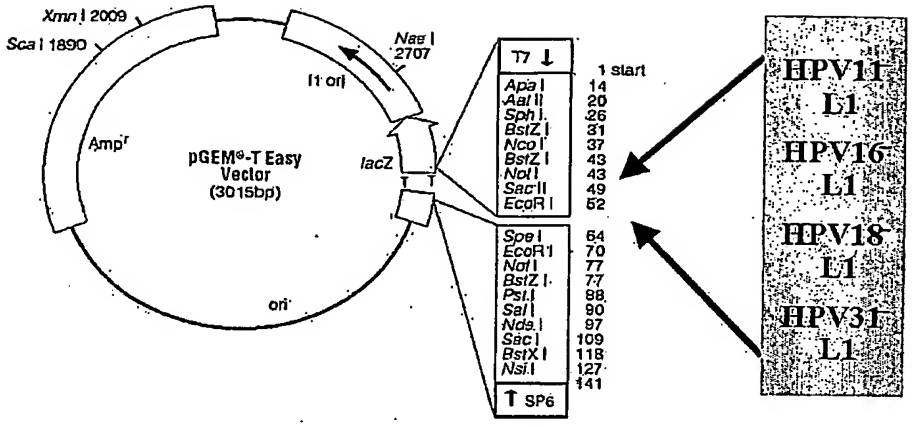
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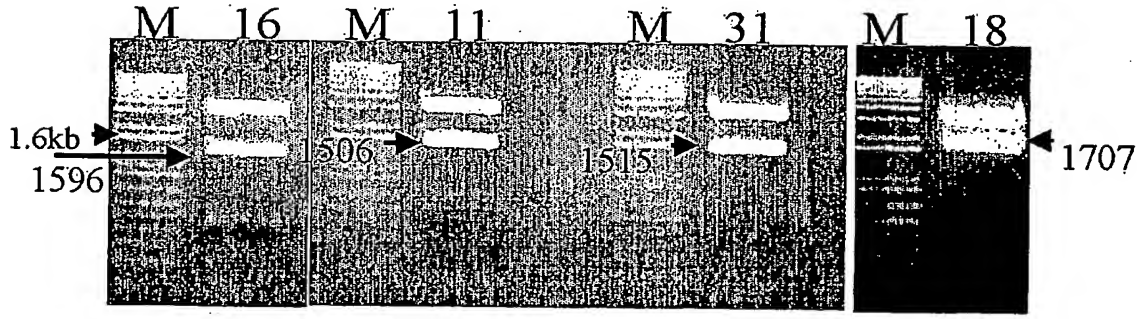
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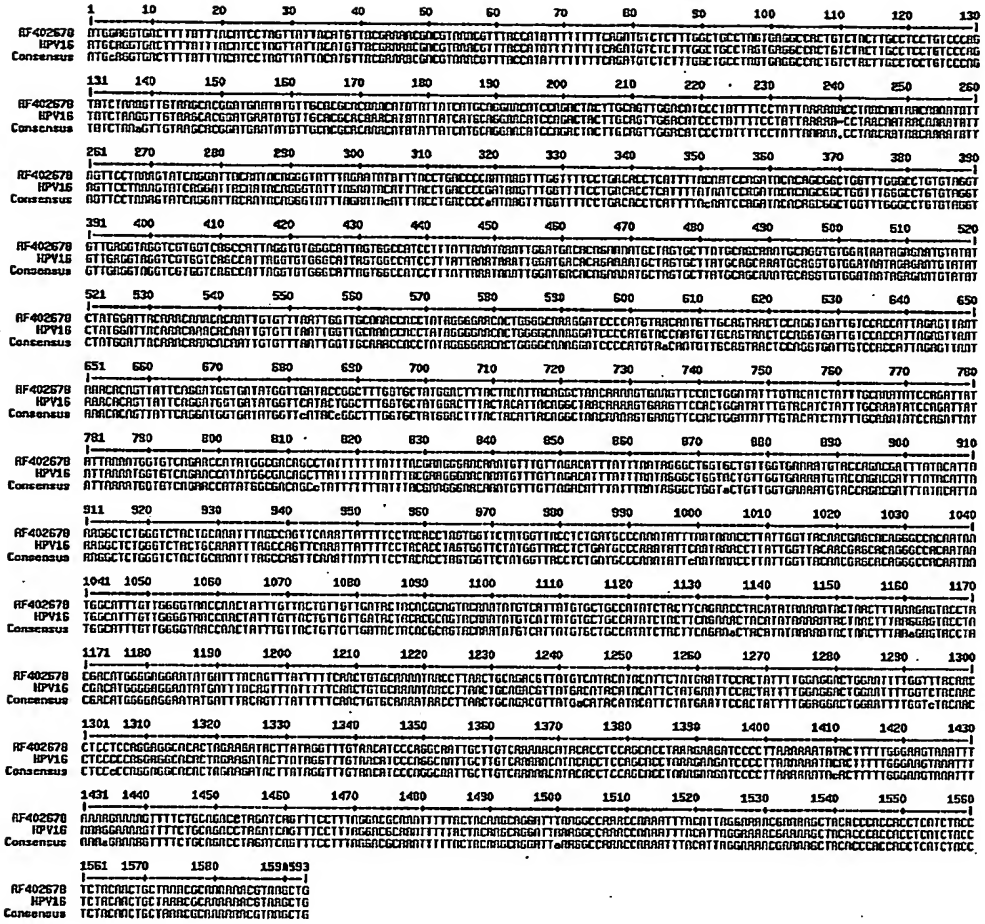
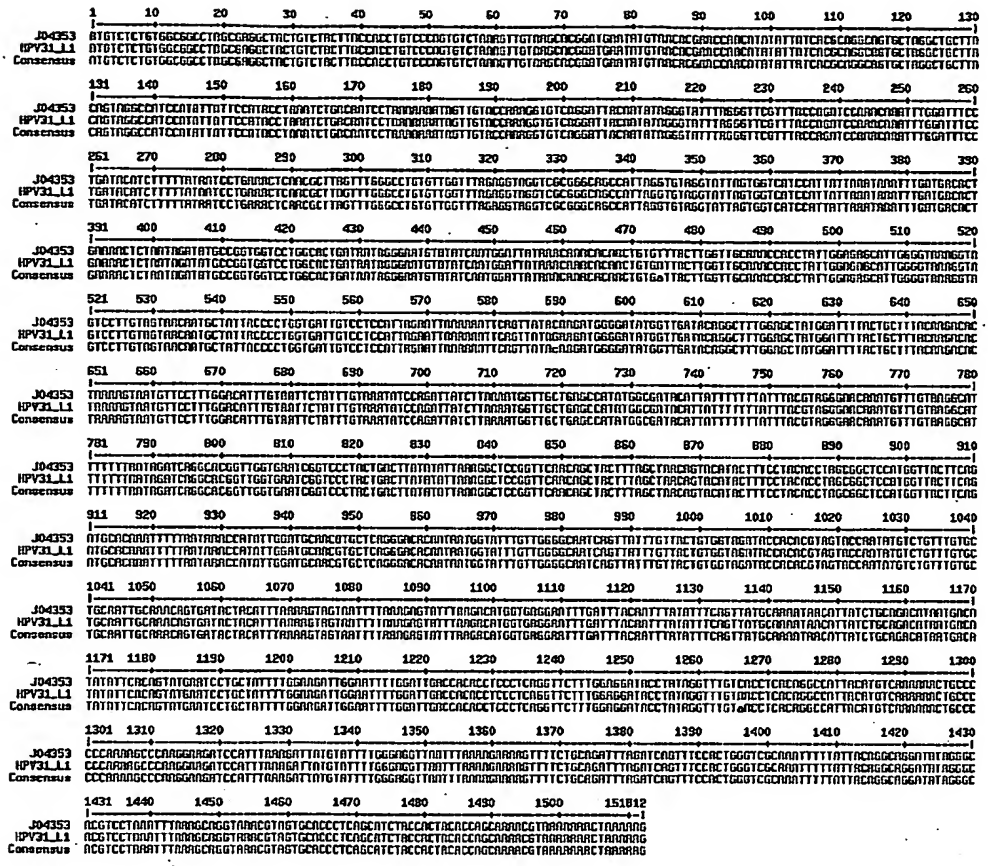


Fig. 3



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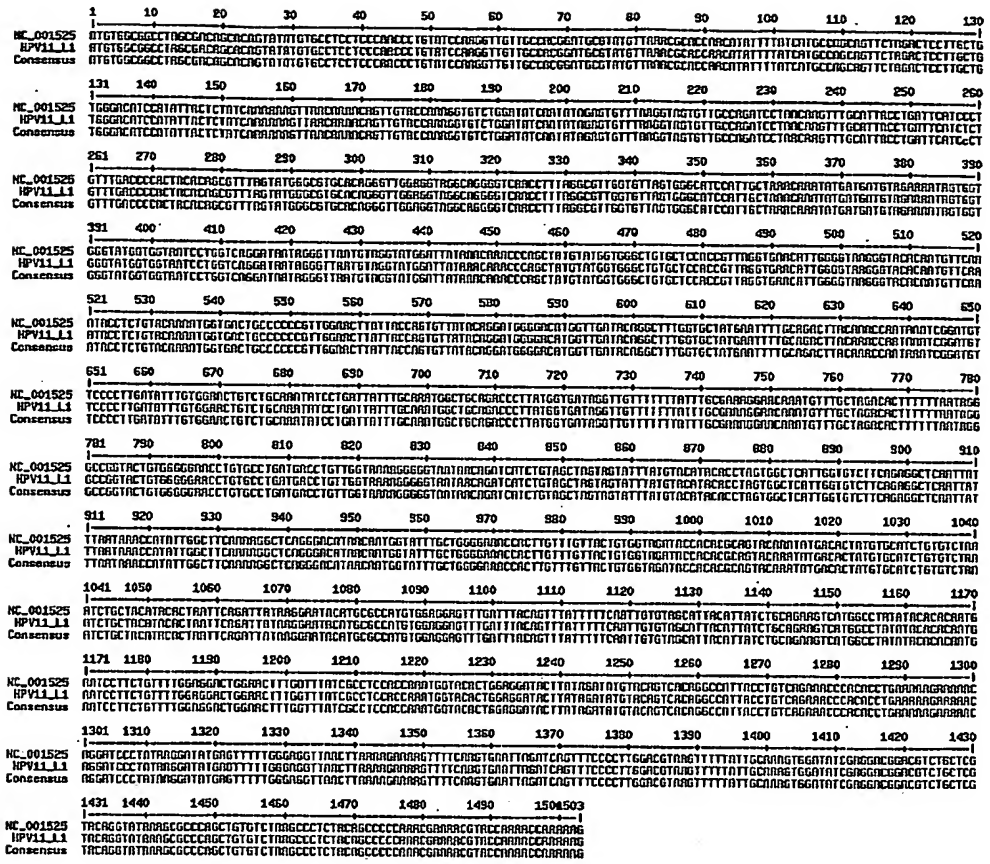


Fig. 5

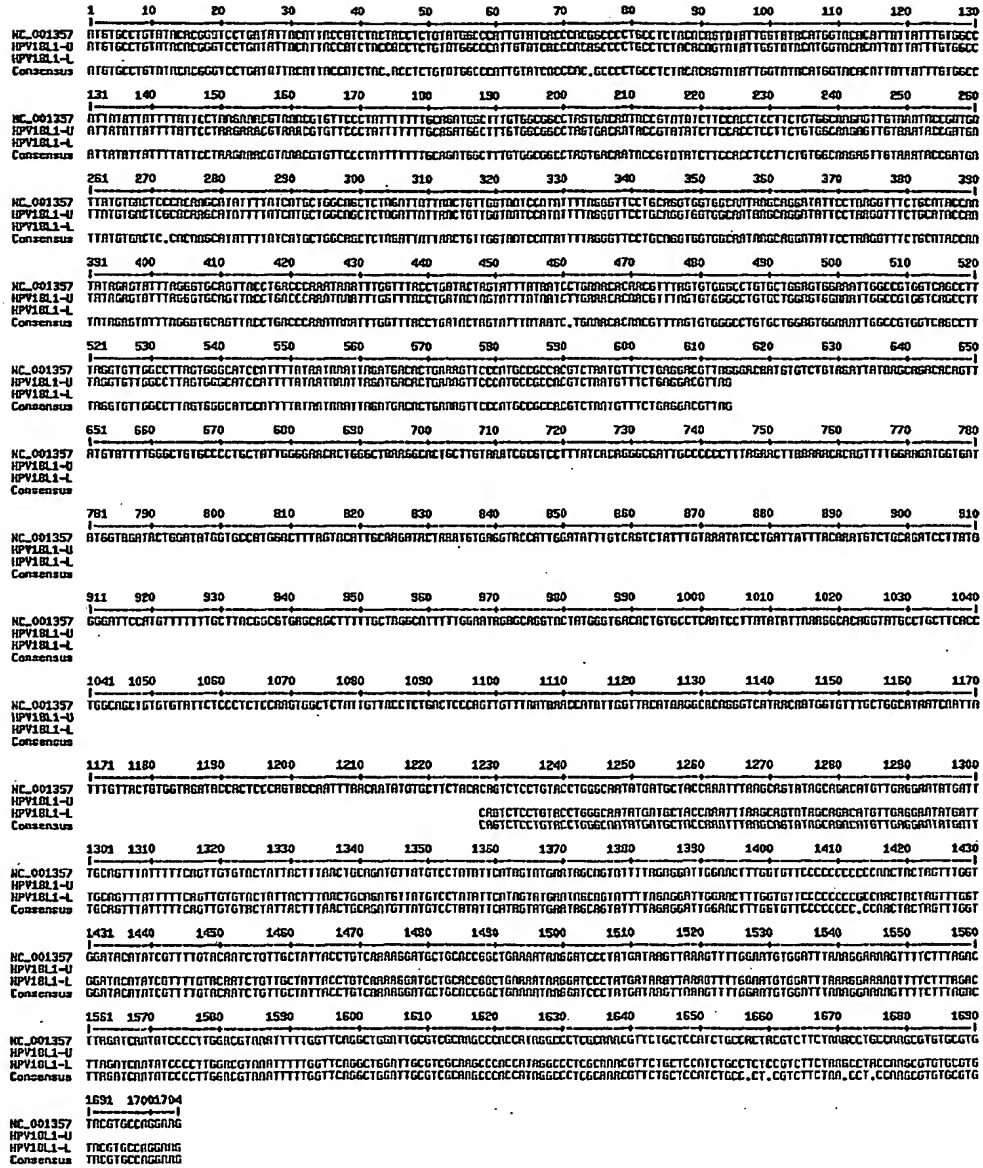


Fig. 6

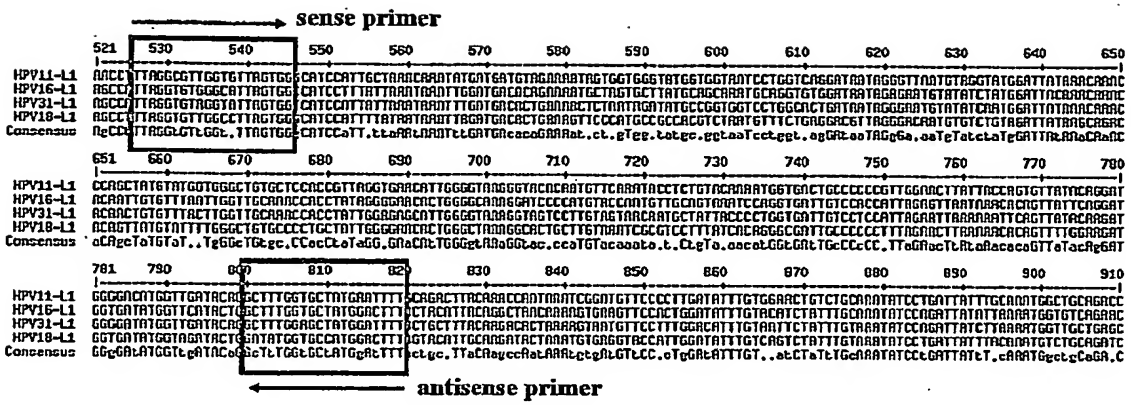
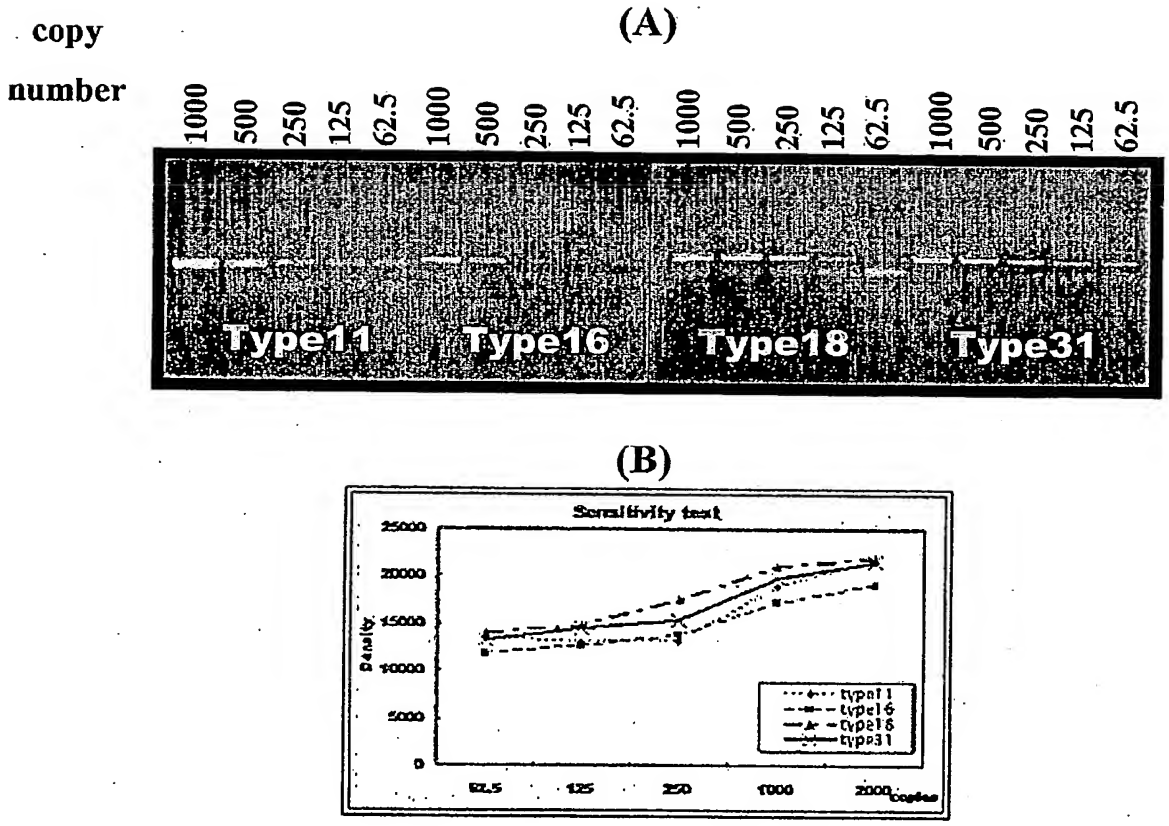


Fig. 7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type16
type18
type31

Fig. 8

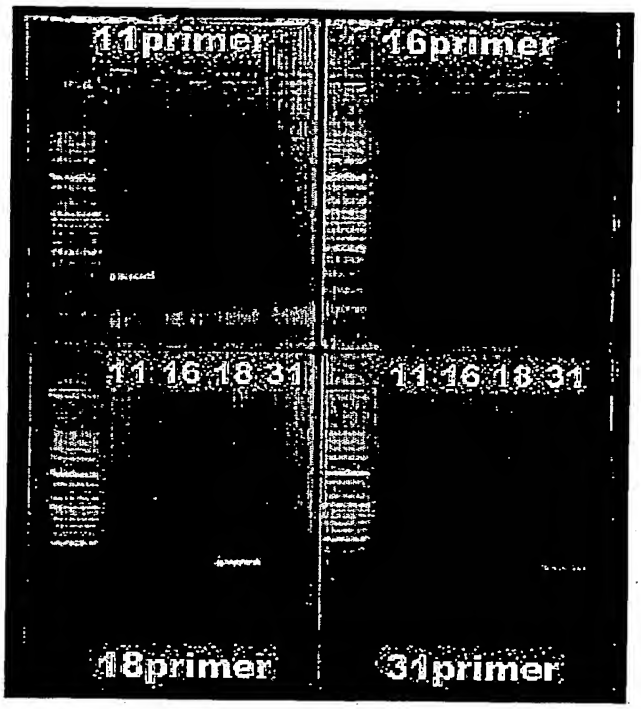
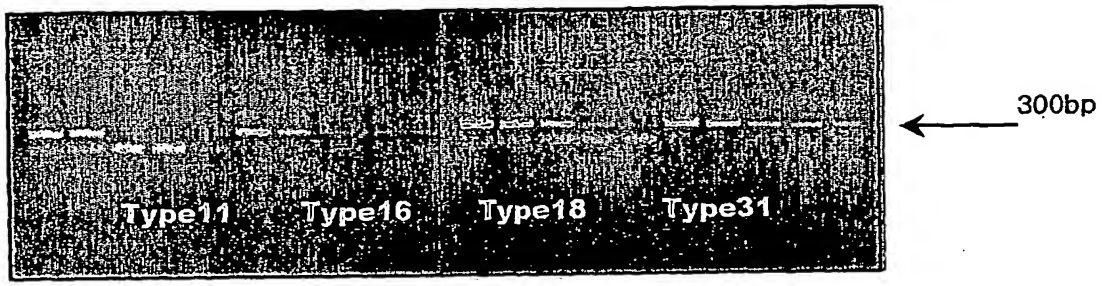


Fig. 9

a. Time point : 0 week



b. Time point : 3 week

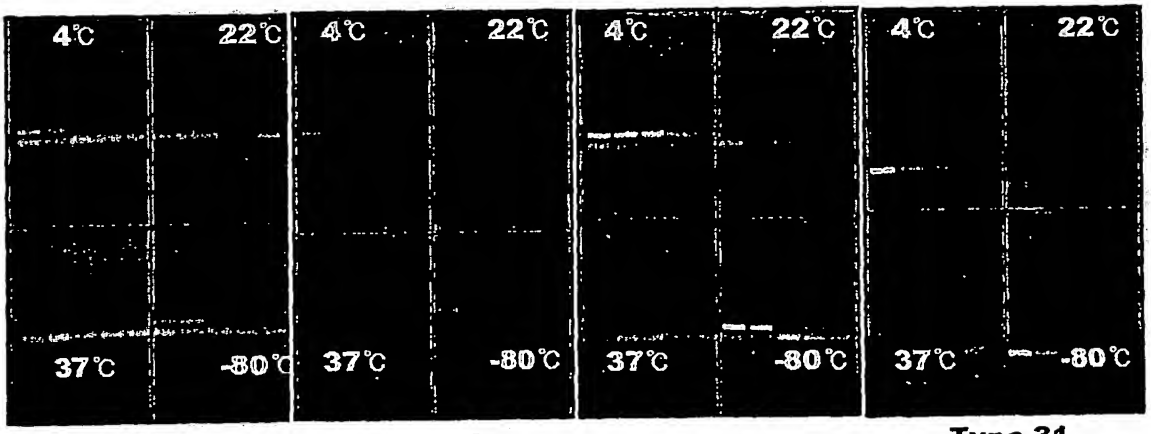
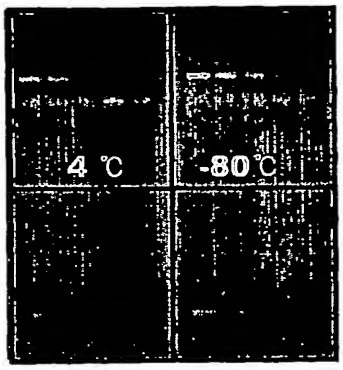


Fig. 10



Type11 : 4 ℃ & -80℃

Y=3.2082X +7596.062
R=0.95

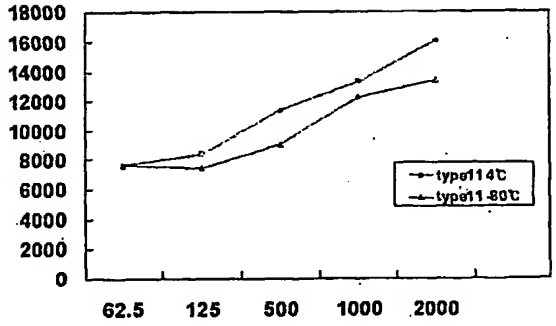
Y=4.219X +8242.484
R=0.97

Type16 : 4 ℃ & -80℃

Y=1.888X +7370.587
R=0.99

Y=2.926X +7273.275
R=0.99

Type11 & Type16



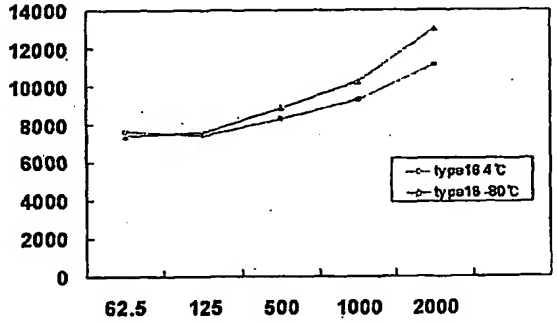
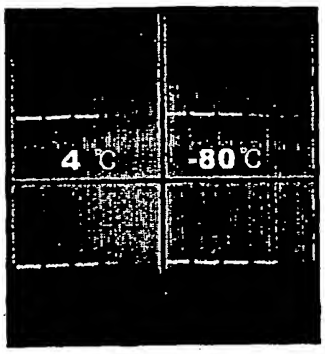


Fig. 11



Type18 : 4 ℃ & -80℃

Y=5.705X +10164.36
R=0.95

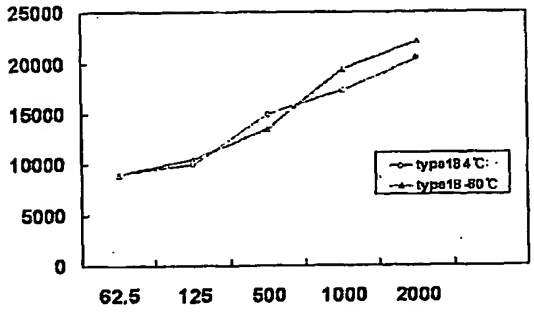
Y=6.7899X +9894.448
R=0.97

Type31 : 4 ℃ & -80℃

Y=4.6447X +8066.789
R=0.94

Y=6.187X + 8781.618
R=0.92

Type18 & Type31



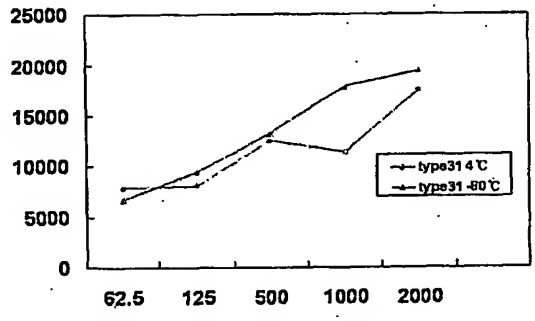
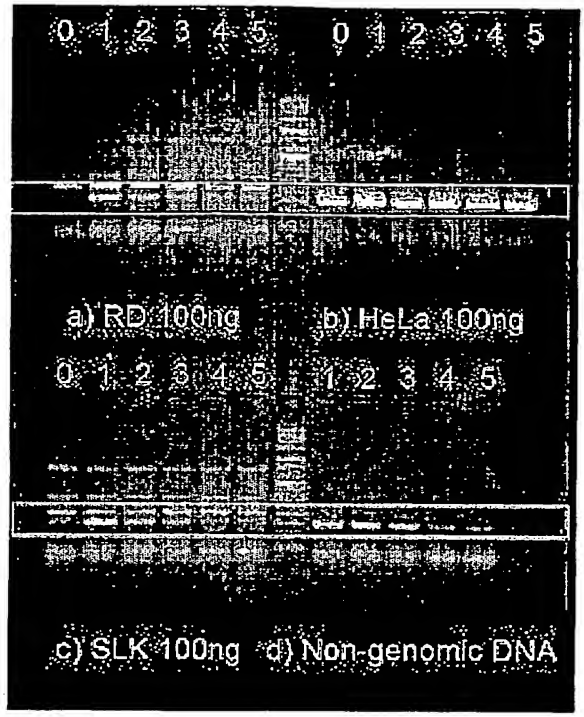


Fig. 12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0 1 2 3 4 5
a) RD 100ng
b) HeLa 100ng
0 1 2 3 4 5
1 2 3 4 5
c) SLK 100ng
d) Non-genomic DNA

<110> Sungkyunkwan university

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/000915

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC8 C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI PubMed, NCBI GenBank, eKIPASS "HPV, L1, high-risk probe, genotyping, etc."

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KR 2004/0036318 A (GENOMICTREE, INC., KR) 30 Apr. 2004 - see the whole document	1 - 8
Y	KR 2004/0078506 A (BIOMEDLAB CO., LTD., KR) 10 Sep. 2004 - see the whole document	1 - 8
Y	WO 2001/068915 A1 (BIOMEDLAB CO., LTD., KR) 20 Sep. 2001 - see the whole document	1 - 8
Y	WO 2003/027323 A1 (BIOMEDLAB CO., LTD., KR) 03 Apr. 2003 - see the whole document	1 - 8
Y	KR 2004/0083674 A (BIOCORE CO., LTD., KR) 06 Oct. 2004 - see the whole document	1 - 8

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 JULY 2006 (10.07.2006)	**10 JULY 2006 (10.07.2006)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
SHIN, Kyeong A
Telephone No. 82-42-481-5589

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/000915

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [x] table(s) related to the sequence listing

   b. format of material
   - [x] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [x] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/000915

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 3-7
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 3-7 relate to a method of diagnosing under Rule 39.1(iv), the search has been carried out and based on the alleged effects of the method.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2006/000915

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 2004/0036318 A	30/04/2004	None	
KR 2004/0078506 A	10/09/2004	None	
WO 2001/068915 A1	20/09/2001	AU 200110604 A1	24/09/2001
		CN 1350593 A	22/05/2002
		EP 1263984 A1	11/12/2002
		JP 15527586 A	16/09/2003
		KR 2001091450 A	23/10/2001
WO 2003/027323 A1	03/04/2003	CN 1558954 A	29/12/2004
		EP 1434873 A1	07/07/2004
		JP 17503177 A	03/02/2005
		KR 2003027178 A	07/04/2003
		US 20040265794 A1	30/12/2004
KR 2004/0083674 A	06/10/2004	None	